Exhibit 7

Activation of Signal Transducer and Activator of Transcription 5 is Required for Progression of Autochthonous Prostate Cancer: Evidence from the Transgenic Adenocarcinoma of the Mouse Prostate System

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ABSTRACT

The constitutive activation of signal transducer and activator of transcription (STAT) proteins has been demonstrated in many diverse human cancer cell lines and clinical tumors including prostate cancer. The STAT family has at least seven members, and the two forms of the STAT5 protein, STAT5A and STAT5B, exhibit a high degree of sequence similarity. We have reported previously that expression of STAT5B, but not STAT5A, could enhance the transforming potential of v-src and induces cell cycle progression and motility in fibroblasts. In the current study we demonstrate specific activation of STAT5B in epithelial cells representing invasive and metastatic prostate cancer. We also demonstrate that the naturally occurring dominant-negative isoform STAT5ΔB can block cell cycle progression through G_1 and inhibit the growth, invasive potential, and clonogenic ability of these prostate cancer cell lines. Furthermore, we report that the dominant-negative isoform STAT5ΔB can inhibit the growth of prostate cancer cells in grafting studies. These results support our hypothesis that specific activation of STAT5, in particular STAT5B, facilitates the progression of prostate cancer.

INTRODUCTION

Prostate cancer is now the second leading cause of cancer death among men in the United States (1). Although surgery is a highly effective therapy for organ-confined disease, there are no curative therapies for the advanced invasive, metastatic, and hormone-refractory forms of prostate cancer. As a consequence, there is an urgent need to identify and characterize the molecular basis of prostate cancer progression. To this end, we have been studying the activation of protein tyrosine kinase signaling cascades by polypeptide growth factors (2). Many groups, including ours, have demonstrated that polypeptide growth factors can act through their cognate receptor tyrosine kinases to regulate not only the differentiation and growth of the prostate gland but also the initiation and progression of prostate cancer.

Recent studies have suggested that prolactin may also play a role in the etiology of benign and malignant prostate disease (3–6). In fact, expression of prolactin receptors in human and rodent prostate glands has been well established (7). In contrast to the membrane-bound tyrosine kinase receptors, the receptor for the peptide hormone prolactin has no intrinsic tyrosine kinase activity and signals through a mechanism mediated by signal transducer and activator of transcription (STAT). There are at least seven STAT proteins in mammalian cells (8), and these transcription factors are activated on tyrosines and serines by phosphorylation.

The primary mechanism of STAT protein activation was originally defined to be via cytokine receptor-mediated activation of the Janus-activated kinase family of tyrosine kinases. However, more recent reports have demonstrated STAT activation by nonreceptor proto-oncogenic tyrosine kinases, including v-src (9–11), v-abl (11, 12), lyn (13), lsk (14), bcr/abl (15), and fes (16). After phosphorylation on a single tyrosine residue (located approximately at amino acid 700), STAT proteins dimerize and translocate to the nucleus, where they bind to specific DNA response elements and interact with other transcription factors to regulate target gene expression. Interestingly, the constitutive activation of STAT proteins has been described in many human tumor cell lines (17–20); however, the identity of the responsible kinase has not always been clear.

Although STAT3 activation is necessary and likely sufficient to transform mammalian fibroblasts (21), the nature of the association between STAT3 and prostate cancer still remains somewhat controversial (22–26) because STAT3 activation has been reported to both stimulate and inhibit the proliferation of the LNCaP prostate cancer cell line (22, 24). Clearly, there is a need to better understand the role of the STAT family of proteins in cancers of epithelial origin.

The STAT5 protein was originally described as a prolactin-inducible mammary gland factor that regulated transcription of the gene encoding the milk protein β-casein (27). We now understand that there are actually two closely related *STAT5* genes, *STAT5A* and *STAT5B*, that encode protein products with 93% identity at the amino acid level. In previous studies, we reported that STAT5B contains a unique sequence that may be a *src/abl* kinase recognition site, and immediately proximal to this site we also identified a putative nuclear localization domain (11). Given their high degree of sequence conservation, it was somewhat surprising that STAT5B, but not STAT5A, could translocate to the nucleus after *Src*-mediated activation and act to enhance the transforming potential of v-src to induce cell cycle progression and cell motility (28). We have also shown that a naturally occurring COOH-terminal truncated form of STAT5 lacking the transactivation domain (STAT5Δ) could act in a dominant-negative fashion to suppress v-src-mediated cell transformation (28, 29). A functional cooperation among androgen receptor, prolactin, and STAT5 has also been reported previously (30).

To further elucidate the role of STAT5 activation in prostate cancer, we undertook this study to examine the consequence of STAT5 expression and activation in the clonal C1A, C1D, and C2H epithelial cell lines derived from a primary prostate tumor in the spontaneous and autochthonous transgenic adenocarcinoma of the mouse prostate model (31). These three lines were chosen because, taken together, they represent a model of prostate cancer progression as follows: C1A cells are immortal but are not tumorigenic; C1D cells are tumorigenic but not metastatic; and C2H cells are both tumorigenic and metastatic. In this report, we demonstrate that even though all of the cell lines express STAT5 protein, we were only able to detect activated STAT5 in the metastatic C2H cell line. Furthermore, enforced expression of the COOH-terminal truncated form of STAT5B lacking the transactivation domain (STAT5ΔB) was able to block cell cycle progression through G_1 and significantly decrease cell growth and the clonogenic potential of C2H cells. We also report that expression of STAT5ΔB was able to inhibit invasive potential of the C2H cells *in vitro* and the

Exhibit 7

ability of these cells to form tumors in nude mice. Clearly, these results demonstrate a correlation between the activation of STAT5 (particularly STAT5B) and prostate cancer progression and identify STAT5B as a target for therapeutic intervention.

MATERIALS AND METHODS

Plasmids. The cDNAs encoding STAT5B, STAT5ΔB, and STAT5A were cloned into the bicistronic vector pEFIRES-N (32) as reported previously (28). v-*src* cDNA was kindly provided by Dr. Hiromitsu Hanafusa and Dr. D. Besser (The Rockefeller University, New York, NY) and cloned into pEFIRES-P vector (32). Bicistronic vectors were kindly provided by Dr. Steve Hobbs (Center for Cancer Therapeutics, Institute of Cancer Research, London, United Kingdom).

Tissue Culture. Clonal Transgenic adenocarcinoma of the mouse prostate C1A, C1D, and C2H cell lines were grown in DMEM (JRH Biosciences) supplemented with 5% fetal bovine serum and 5% Nu-Serum. LipofectAMIN-Eplus reagent (Life Technologies, Inc.) was used for all transfections. Transformants were selected with 800 ng/ml G418 sulfate (Geneticin; Life Technologies, Inc.). Human LNCaP and PC-3 prostate cancer cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum.

Generation of Stable Cell Lines. We used the bicistronic vectors pE-FIRES-N or pEFIRES-P (32) to facilitate studies and determine the functional consequences of enforced expression of STAT5 in prostate cells in culture. These vectors were engineered such that the specific STAT5 isoform and the antibiotic resistance gene (to neomycin or puromycin) would be expressed from a single transcript driven by the human polypeptide chain elongation factor-1a promoter. Placing the *STAT* cDNA after an internal ribosome entry site (IRES) ensured that clones resistant to the selected antibiotic would also express high levels of STAT5 isoform. Using this strategy, we generated stable clones expressing STAT5B and STAT5ΔB. As a control, we used a C2H cell line stably transfected with IRES-N vector. Expression of the corresponding proteins was confirmed by Western blot analysis.

Preparation of Whole Cell Extracts. Cells were grown in complete media until 80% confluence and then grown for 16 h in media supplemented with 5% charcoal-stripped fetal bovine serum. All were then rinsed twice with ice-cold PBS (Invitrogen Corp., Grand Island, NY) and scraped into radioimmunoprecipitation assay buffer [50 mM NaF, 10 mM Na$_4$P$_2$O$_7$, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40, 150 mM NaCl, 9.1 mM Na$_2$HPO$_4$, and 1.7 mM NaH$_2$PO$_4$ (pH 7.4)] containing the following protease and phosphatase inhibitors: 1 mM Na$_3$VO$_4$; 1 mM phenylmethylsulfonyl fluoride; 2 μg/ml aprotinin; 2 μg/ml antipain; 2 μg/ml leupeptin; and 2 μg/ml benzamidine. After incubation at 4°C for 30 min, cell extracts were recovered by centrifugation, and supernatants were collected. Protein concentrations were measured using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA).

Western Blot Analysis. Protein samples were separated by 7.5% SDS-PAGE and transferred overnight to an Immunobilon-P membrane (Millipore Corp., Bedford, MA). We routinely analyzed 60 μg of total cell lysate/lane. The specific polyclonal antibodies to the COOH-terminal region of STAT5A and STAT5B and to the activated form that is phosphorylated on tyrosine 700 were described previously (11, 29). NH$_2$-terminal STAT5 (N-20) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and β-actin AC-74 antibody was obtained from Sigma (St. Louis, MO).

Cell Proliferation Assay. Cell proliferation assays were performed on stably transfected cells using the colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2*H*-tetrazolium assay (CellTiter 96 AQ; Promega, Madison, WI) according to the manufacturer's protocol. Cells were plated in 98-well plates at a density of 2 × 10^3 cells/well. Cells were grown for 12 h in complete media followed by 36 h in serum-free media.

Clonogenic Assay. Stable transformants (200 cells) were plated in 100-mm dishes for 10 days in regular medium. Dishes were washed once with 0.85 M NaCl, stained with crystal violet (Sigma), dissolved in ethanol for 15 min, and washed. Colonies containing >50 cells were scored as positive. Each experiment was performed twice in duplicate. A control cell line transfected with IRES-N was used to establish a baseline.

Cell Cycle Analysis. Cell cycle distribution experiments were performed with an EPICS XL flow cytometer (Beckman Coulter). A total of 3 × 10^5 to

5 × 10^5 cells were recovered by centrifugation and resuspended in 0.5 ml of PBS. During mixing, 5 ml of ice-cold ethanol were added. The cell suspension was incubated overnight at 4°C. Fixed cells were recovered by centrifugation and resuspended in 0.75 ml of PBS. RNase A (Boehringer Mannheim, Indianapolis, IN) was added to a final concentration of 0.1 mg/ml and incubated at 37°C for 30 min. After the addition of propidium iodide (Boehringer Mannheim) to a final concentration of 0.05 mg/ml, cells were analyzed by flow cytometry (fluorescence-activated cell sorting) for their DNA content. The distribution of cells in different phases of the cell cycle was quantitated (Beckman Coulter System II version 3.0) and represented as the percentage of cells in S-G$_2$ phases. The experiment was run in duplicate and repeated twice.

Electrophoretic Mobility Shift Assays. The electrophoretic mobility shift assays were performed as described previously (29). Briefly, 10 μg of whole cell extract and 2–4 μg of poly(deoxyinosinic-deoxycytidylic acid) were incubated on ice for 15 min. ^{32}P-labeled γ-activated site probe from β-casein promoter (0.5 ng) was then added, followed by a further incubation on ice for 15 min. The entire binding reaction was then loaded onto a pre-electrophoresed (30 min), 5% polyacrylamide gel (19:1 acrylamide to bisacrylamide ratio) containing 2.5% glycerol and electrophoresed at 10 V/cm in 0.25× Tris-borate-EDTA and exposed to XAR-5 film (Kodak, Rochester, NY).

Generation of a Conditional Inducible Form of STAT5. Our current understanding of the activation of STAT5 protein is that phosphorylation is the consequence of an activation of an endogenous tyrosine kinase. To further study the biological consequence of STAT5B action, it became necessary to isolate STAT5B activation from other cellular events. To this end, we exploited a conditional chemical induced dimerization (CID) system to specifically be able to control the activation of STAT5ΔB. This system exploits the property of a naturally occurring molecule called FK506 to bind to FK506-binding protein-12 (33). Because the interaction of FK506-binding protein-12 with FK506 occurs at a stoichiometry of 1:1, two FK506 compounds were chemically tethered, resulting in a bivalent drug (FK1012) with the capacity to interact with and essentially force the dimerization of molecules harboring FK506-binding protein-12 domains.

Using CID technology, a construct was generated to encode a STAT5ΔB fused at the COOH terminus to FK506-binding protein-12-binding domain (Fv) that was followed by an additional hemagglutinin (HA) epitope sequence. To prevent spontaneous dimerization and nuclear translocation of the STAT5ΔB construct, tyrosine 699 was specifically changed to a phenylalanine residue. A sequence encoding the jellyfish green fluorescent protein (GFP) was also placed after the HA epitope to further facilitate detection of the modified STATΔ5B protein. We used the third-generation CID AP20187 (34) to induce dimerization and activation of our construct.

Once generated, the STAT5ΔB^{Y699F}FvHA-GFP construct was introduced into C2H and C1A cells, and stable lines were established. To test the ability of the CID-based system to activate STAT5ΔB, we treated cells harboring the STAT5ΔB^{Y699F}FvHA-GFP construct with AP20187 for 5 min. As shown in Fig. 1, we were able to monitor the subcellular location of the fusion protein by GFP and observed a rapid translocation of the STAT5ΔB^{Y699F}FvHA-GFP to the nucleus. To demonstrate the functional ability of dimer-activated STAT5ΔB^{Y699F}FvHA-GFP, we performed electrophoretic mobility shift assays with specific DNA consensus sequences for STAT5 derived from the β-casein promoter γ-activated site element (Fig. 1C). We were unable to detect any activity of the STAT5ΔB^{Y699F}FvHA-GFP fusion protein in the absence of AP20187. On the basis of this observation, not only were we able to construct a conditional allele of STAT5ΔB that translocated to the nucleus specifically after stimulation with AP20187, but we were also able to demonstrate that such a conditionally activated allele functionally recognized and bound a known STAT5B target DNA sequence.

Cell Invasion Assay. Approximately 5 × 10^4 stably transfected C2H cells were added to each of BioCoat extracellular matrix (ECM) cell culture inserts (24-well size; 6.4-mm diameter; Becton Dickinson Labware, Bedford, MA) and an equal number of control inserts (without ECM) in serum-free culture media. Media supplemented with 10% serum served as a chemoattractant in the lower compartment. After 12 h, the noninvasive cells were removed with a cotton swab, and cells that had migrated through the porous insert and adhered to the lower surface were fixed in 4% formaldehyde and stained using 0.5% crystal violet. The membranes were then removed and mounted on slides. Cells from five adjacent diagonal fields, each covering 0.25-mm^2 areas, were counted at ×200 magnification. Data are expressed as the percentage of

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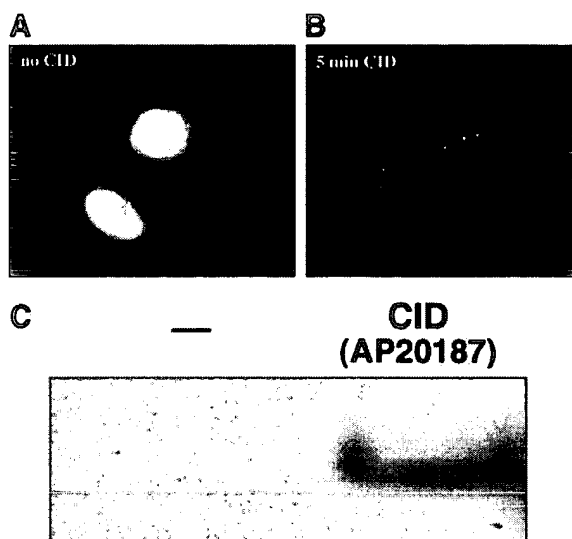
B

C — CID (AP20187)

Fig. 1. Characterization of the inducible STAT5ΔB^{Y699F}FvHA-GFP. *A*, the STAT5ΔB^{Y699F}FvHA-GFP is not activated and is localized in the cytoplasm in the absence of the dimerizer molecule AP20187. *B*, STAT5ΔB^{Y699F}FvHA-GFP is activated and translocated to the nucleus in response to AP20187. *Green*, STAT5ΔB^{Y699F}FvHA-GFP; *blue*, nuclear staining by 4',6-diamidino-2-phenylindole. *C*, electrophoretic mobility shift assay demonstrates the ability of STAT5ΔB^{Y699F}FvHA-GFP to shift a γ-activated site probe from β-casein promoter in the presence of the CID AP20187.

invasion through the Matrigel matrix membrane relative to the migration through the control membrane (not covered with ECM). The experiment was performed twice in triplicate.

Tumorigenicity of Cell Lines. Cells were grown to 95% confluence in regular media, washed three times with serum-free medium, treated with trypsin, washed with 5-fold excess media to neutralize trypsin, and recovered by centrifugation. The live cells were then resuspended in serum-free medium at 7.5 × 10⁷ cells/ml and s.c. injected in a volume of 0.2 ml into the hindquarters of male nude mice (1.5 × 10⁶ cells) using a 21-gauge needle. The injection site was pinched for 1 min to prevent any cell suspension from leaking out. Animals were maintained on standard diet and monitored three times a week for tumors starting 2 weeks after injection. Animal care was in accordance with the guidelines of the NIH Animal Research Advisory Committee.

Statistical Analysis. Values were expressed as the mean ± SE. Statistical analyses were performed by Student's *t* test for paired comparisons using StatView program (Abacus Concepts, Inc., Berkeley, CA). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Expression and Activation of STAT5 in Prostate Cancer Cell Lines.

We have used a series of related murine prostatic epithelial tumor cell lines to investigate the consequence of expression and activation of STAT5 during the natural history of prostate cancer. These epithelial cell lines were derived from a primary prostate tumor of a single transgenic adenocarcinoma of the mouse prostate mouse. Although they do not express T antigen, they all express androgen receptor. For comparison, we also examined STAT5 expression in the human prostate cancer cell lines LNCaP and PC-3.

To test the hypothesis that expression and activation of STAT5 are associated with prostate cancer progression, we performed immunoblot studies with an NH₂-terminal antibody that recognizes both STAT5A and STAT5B isoforms. As shown in Fig. 2, we detected multiple processed forms of STAT5 in all samples as well as a COOH-terminal truncated dominant-negative isoform of STAT5 (STAT5Δ) in C1A and C1D cell lines (Fig. 2A). When we used a STAT5A-specific antibody, we detected STAT5A in C1A, C1D, C2N, and LNCaP cells but were unable to detect STAT5A expression

in C2H and PC-3 cells (Fig. 2B). When we used a STAT5B-specific antibody, we detected expression of STAT5B in all cell lines except C1A (Fig. 2C).

Next, we determined the levels of activated STAT5 protein in the cells using p5^{700Y} antibody directed to the phosphotyrosine at position 700 required for STAT5 dimer formation and DNA binding (29). As shown in Fig. 2D, we were only able to detect activated full-length STAT5 in extracts prepared from the metastatic C2H and C2N cell lines and in both human prostate cancer lines. Interestingly, C1A and C1D cell lines also express an activated short form of STAT5 that corresponds to the dominant-negative isoform of STAT5 (STAT5Δ). Unfortunately, the antibody could not discriminate between STAT5A and STAT5B, and the phosphorylated form of STAT5B can appear to migrate with the same mobility as STAT5A. Nevertheless, we have demonstrated the differential expression and activation of the STAT5 isoforms in the prostate cell lines and that the more aggressive cell lines expressed the highest levels of phosphorylated STAT5 (tyrosine 700).

Consequence of Deregulated Expression of STAT5 in Prostate Cancer. To elucidate the consequence of deregulated expression of STAT5 in prostate cancer, we next generated stable cell lines expressing high levels of either STAT5B or a dominant-negative isoform of STAT5B lacking the COOH-terminal transactivation domain (Fig. 3). As shown in Fig. 4, enforced expression of STAT5B did not result in a significant increase in growth rate in C2H cells that already ex-
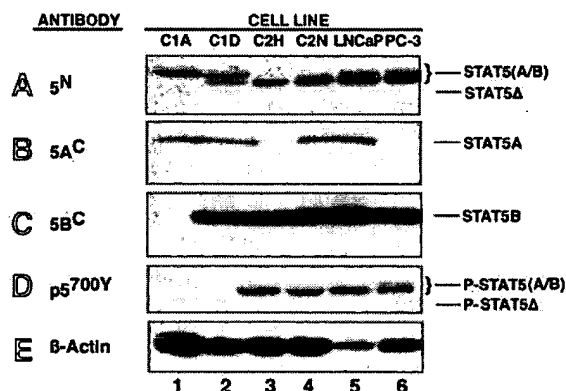

Fig. 2. Level of specific tyrosine phosphorylation of STAT5 in prostate cell lines. Extracts prepared from C1A, C1D, C2H, C2N, LNCaP, and PC-3 cell lines were analyzed by direct Western blot with the following: *A*, 5^N, an antibody directed against the NH₂-terminal of STAT5 that recognizes both STAT5A and STAT5B isoforms; *B*, 5A^C, an antibody directed against the unique COOH-terminal epitope of STAT5A; *C*, 5B^C, an antibody directed against the unique COOH-terminal epitope of STAT5B; *D*, p5^{700Y}, an antibody raised against a peptide containing the phosphotyrosine required for STAT5 dimer formation and DNA binding; and *E*, β-actin, an antibody directed against β-actin.
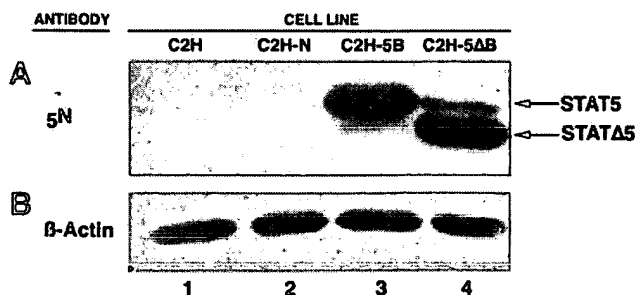

Fig. 3. Expression of STAT5 isoforms in stably transfected C2H cells. Extracts were prepared from untransfected C2H cells (*Lane 1*) and C2H cells stably transfected with control vector IRES-N (*Lane 2*), STAT5B (*Lane 3*), and STAT5ΔB (*Lane 4*). Cell line extracts were analyzed by Western blot analysis using A5^N, an antibody directed against the NH₂-terminal of STAT5 (*A*), and β-actin, an antibody directed against β-actin (*B*).
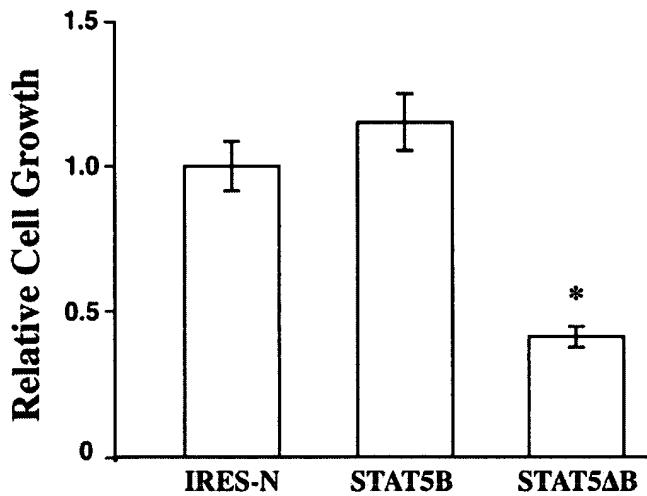
Fig. 4. Cell growth assay. Cell proliferation assays of stably transfected C2H cells were performed as described in the text. Approximately 2×10^3 cells/well were plated in 98-well plates in triplicate for 12 h in complete medium and then seeded for 48 h without serum. The experiment was repeated twice. The relative value of the absorbance of the cells transfected alone with empty vector is 1 (line IRES-N). Each *error bar* indicates the SD. *, $P = 0.0023$.
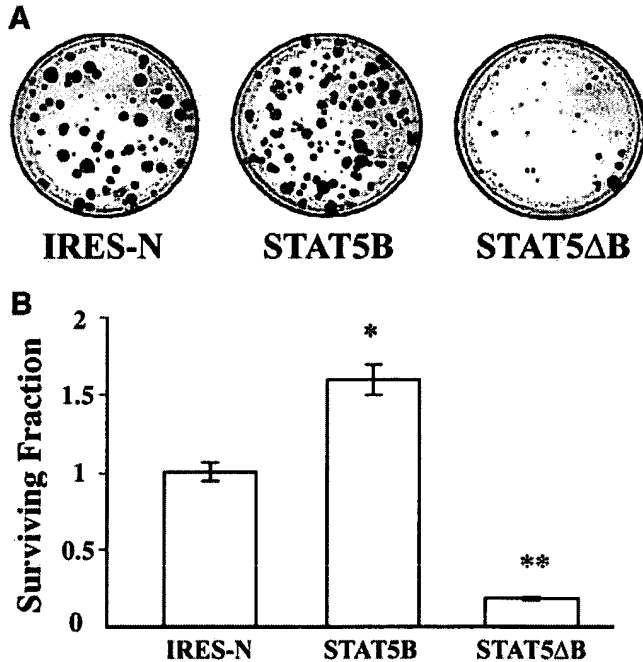
A

IRES-N   STAT5B   STAT5ΔB

B

Fig. 5. Clonogenic assay. Stable transformants (200 cells) were plated in 100-mm dishes for 10 days. Dishes were washed once with 0.85 M NaCl, stained with crystal violet (Sigma), dissolved in ethanol for 15 min, and washed (Fig. 4A). Colonies containing >50 cells were scored as positive. Each experiment was performed in duplicate and repeated twice. Viable colonies were counted using the control cell line (IRES-N) as baseline. *Error bars*, SD (Fig. 4B). *, $P = 0.006$; **, $P = 0.0005$.

We next examined the consequence of enforced expression of STAT5B or STAT5ΔB on transformation and survival. As shown in Fig. 5, enforced expression of wild-type STAT5 increased the clonogenic potential of C2H cells by approximately 60%, whereas enforced expression of STAT5ΔB suppressed this potential by 80%. As shown in Fig. 6, after 48 h of serum deprivation, enforced expression of STAT5B resulted in a 20% increase in the number of C2H cells in S-G₂, compared with cells carrying an empty vector. At the same time, enforced expression of STATΔ5B blocked cell cycle progression by 50%. Taken together, these data clearly support a role for STAT5 in cell cycle regulation and cell survival and also support the hypothesis that STAT5 is required for prostate cancer cell viability and transformation.

In Vivo **Studies.** To test the ability of STAT5B to contribute to tumorigenicity of prostate cancer *in vivo*, we engrafted C2H cells carrying either empty vector (controls) or the STAT5B or STAT5ΔB constructs into nude mice. As shown in Fig. 7, we were unable to detect a statistically significant difference between the growth of cells expressing STAT5B and that of the control cells. However, the cells expressing STAT5ΔB were unable to grow and develop tumors even after 2 months of incubation. Clearly, expression and activation of STAT5B are an important signal that profoundly influences the ability of cells to establish and grow as tumors in animal studies.

Dominant-Negative Isoform of STAT5B Blocks Invasive Potential of Prostate Cancer Cells. The acquisition of invasive potential is generally regarded as a feature of prostate cancer progression toward malignancy and is a requisite property for metastasis. To test the ability of the various STAT5 isoforms to influence the invasive potential of prostate cancer cells, we measured the ability of stably transfected and control cells to invade through a Matrigel basement membrane matrix. This matrix was extracted from the Engelbreth-Holm-Swarm mouse tumor. To display invasive potential in this
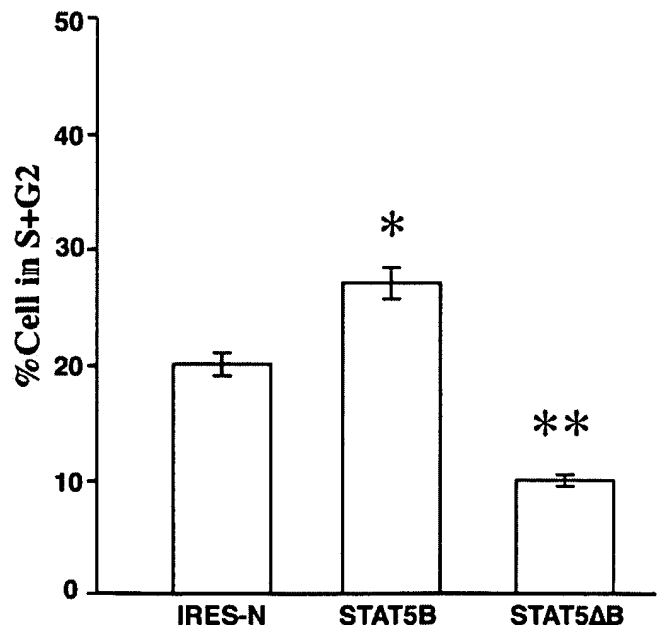

Fig. 6. Cell cycle analysis. Stably transfected C2H cells with various combinations of different forms of STAT5 and IRES-N vector alone (see above) at 70-80% of confluence were serum starved for 48 h. After the indicated time, cells were fixed with ethanol and stained with propidium iodide. The cell populations were analyzed for DNA content using a flow cytometer (EPICS XL; Beckman Coulter). The cell cycle distribution of the profiles was quantitated with the computer program for this flow cytometer (Beckman Coulter System II version 3.0) and is shown in a *bar diagram* presenting the percentage of cells in S-G₂ phases. The experiment was repeated twice, each in duplicate. *, $P = 0.002$; **, $P = 0.0004$.

pressed the highest levels of endogenously activated STAT5B. It was interesting that the level of activated STAT5B was not found to increase in these cells (data not shown), suggesting that the level of activated STAT5 was tightly regulated and/or limited by an intrinsic cellular mechanism. In contrast, enforced expression of STAT5ΔB significantly decreased C2H cell growth by >50%. We observed a similar result in the C2N cell line, which is also tumorigenic and metastatic (data not shown). From these observations, it appears that cell proliferation was dependent on STAT5B activation and that enforced expression of STAT5ΔB could specifically block this signal.
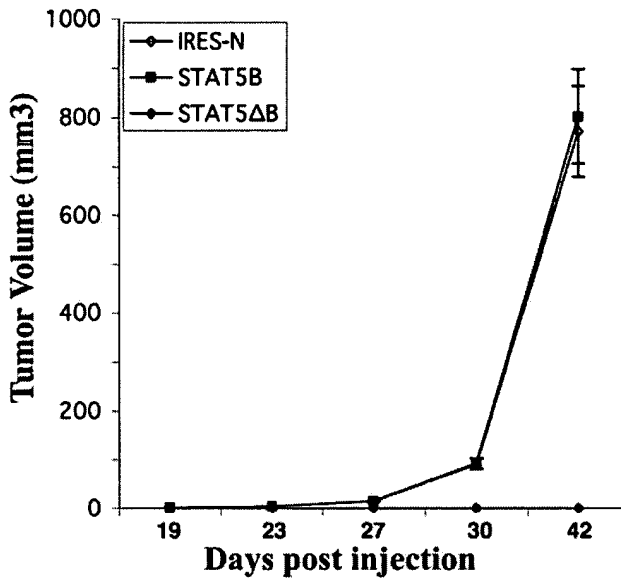
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Fig. 7. Tumor growth *in vivo*. Male nude mice (5 animals/condition in two separate experiments) received injection s.c. in each flank with 1.5×10^6 stably transfected C2H cells. Tumor growth was monitored every 3–7 days using digital calipers. To estimate the tumor volume, we used the following formula: $4\pi/3 \times (\text{width}/2)^2 \times (\text{length}/2)$, representing the three-dimensional volume of an ellipse.

assay, a cell had to (*a*) attach to the ECM, (*b*) degrade the ECM, and (*c*) migrate into ECM. As shown in Fig. 8, enforced expression of STAT5B did not significantly influence the invasive potential of C2H cells compared with the controls harboring the empty vector. In contrast, STAT5ΔB was able to reduce invasion of the C2H cells from 42% to 13% (a change of 69%). Although enforced expression of STAT5ΔB^{Y699F}FvHA-GFP alone was sufficient to reduce invasion by 50%, addition of AP20187 reduced invasion by 83%. Clearly, enforced expression of STAT5ΔB was able to significantly reduce the invasive potential of C2H cells, and the CID-conditional STAT5ΔB^{Y699F}FvHA-GFP allele was the most efficacious form of STAT5ΔB tested.

DISCUSSION

It has been suggested that activation of STAT5 is both necessary and sufficient for malignant transformation (35). However, mouse knockout studies have revealed that STAT5 can play either an essential or a nonessential role in oncogenesis (36, 37). A clear understanding of the function of STAT5 *in vivo* has been complicated by the existence of two nearly identical genes, *STAT5A* and *STAT5B*, that are closely linked in both mouse [chromosome 11 (38)] and human [chromosome 17 (39)]. An additional complication stems from the existence of naturally occurring COOH-terminal truncated dominant-negative isoforms of STAT5 (29). Our current study uses a model of spontaneous prostate cancer in mice to provide support for our hypothesis that activation of STAT5B promotes progression of prostate cancer.

Using a panel of related cell lines derived from a primary prostate tumor in the spontaneous autochthonous transgenic adenocarcinoma of the mouse prostate model and human prostate cancer cell lines, we have demonstrated significant levels of activated STAT5 that specifically correlate with tumorigenic and metastatic potential. Interestingly, enforced overexpression of STAT5B by itself had no observable impact on the growth of C2H cells, whereas enforced expression of the dominant-negative STAT5ΔB isoform was able to inhibit growth and clonogenic ability of this tumorigenic and metastatic

prostate cell line. Moreover, STAT5ΔB demonstrated specificity for cells such as C2H that expressed endogenous forms of phosphorylated STAT5, whereas STAT5ΔB was not able to inhibit growth of the STAT5-deficient C1A cell line. Although expression of STAT5 was found to be concomitant with the ability of prostate cells to grow in culture, the activation of STAT5B was specifically identified in cells representing advanced disease.

The specific role of STAT proteins in cell growth and cell cycle regulation is incompletely understood. In our experiments, enforced expression of STAT5B modestly increased cell growth and cell cycle progression, whereas enforced expression of STAT5ΔB significantly decreased cell growth as well as cell cycle progression through G_1. As noted in the text, expression of exogenous STAT5B resulted in only a slight increase in the number of cells in S-G_2 compared with control cells, whereas enforced expression of STATΔ5B blocked cell cycle progression by 50%. These data further support a role of STAT5B in tumor progression but also underscore the fact that simply increasing the level of STAT5 expression does not necessarily increase the ability of cells to grow *in vivo*. This is exemplified by the grafting studies, in which we were unable to observe a statistically significant difference between the growth of cells expressing STAT5B and cells harboring empty vector. Moreover, cells expressing STAT5ΔB were unable to develop into a tumor, likely related to the ability of STAT5ΔB to efficiently inhibit the invasive potential of the C2H cells. The observation that the conditionally activated form of STAT5ΔB was able to significantly inhibit the invasive potential of the C2H cells even in the absence of the AP20187 suggests that STAT5ΔB^{Y699F}FvHA-GFP competes with STAT5B activation in the cytoplasm. However, AP20187-dependent activation of STAT5ΔB^{Y699F}FvHA-GFP was able to specifically induce nuclear translocation and DNA binding, and this likely contributed to the additional inhibition of invasive potential observed. Taken together, these results implicate the specific activation of STAT5B as a crucial event in transformation and tumorigenesis.

Previous reports have demonstrated the direct participation and requirement of STAT3 in tumor growth (17, 21). Recent functional studies have also demonstrated that a dominant-negative isoform of STAT3, like STAT5ΔB, has a profound inhibitory effect on tumorigenic progression, but unlike STAT5ΔB, it had no obvious effect on invasion (40). Reports have also implicated STAT3 in the growth of prostate cancer, but it has been difficult to reconcile all of the data (22, 24). There is, in fact, growing support for a role for STAT5 in cell survival, under which conditions STAT3 induced cell differentiation and apoptosis (41, 42) or
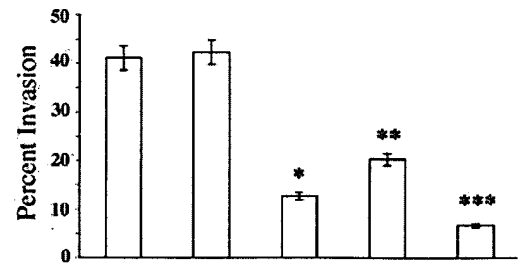
Fig. 8. Cell invasion assay. Stably transfected C2H cells from five adjacent diagonal fields, each covering a 0.25-mm^2 area, were counted at high magnification. Data are expressed as the percentage of invasion through the Matrigel matrix and membrane relative to migration through the control membrane. *, $P < 0.0001$; **, $P = 0.0001$; ***, $P < 0.0001$.

growth arrest and terminal differentiation (43). Moreover, a recent report demonstrated that there is specific activation of STAT5 in prostate cancer cell lines and in human prostate tumor samples and that blocking the STAT5 pathway could induce death of prostate cancer cells (44). Furthermore, a reciprocal pattern of STAT5 and STAT3 activation has been observed during mammary gland development (45), suggesting that these STAT proteins may play different, even antagonistic, or opposite roles, depending on the tissue and cell type. Although recent data have demonstrated that deletion of STAT3 blocks mammary gland involution (46), most studies to date provide strong evidence that STAT5 and STAT3 participate in the development and progression of human cancers by inducing cell proliferation and/or preventing apoptosis (18). Therefore, there is a distinct possibility that STAT5 and STAT3 may actually cooperate in the process of transformation and tumor progression. The possibility also exists that STAT3 is responsible for initiation of tumor formation, whereas STAT5B is required for tumor progression. Although studies designed to test these possibilities are under way, our data clearly support the hypothesis that inappropriate regulation of STAT5 expression and activation facilitates the progression of prostate cancer and identifies STAT5B as a target for therapeutic intervention.

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